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Perinatally Administered Bisphenol A Acts as a Mammary Gland Carcinogen in Rats

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Running Title: BPA as a Developmental Mammary Gland Carcinogen

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ABSTRACT

Background: Environmental exposure to bisphenol A (BPA) affects mammary gland development of rodents and primates. Prenatal exposure to environmentally relevant doses of BPA increased the number of intraductal hyperplasias and ductal carcinomas *in situ* by 50 days of age in Wistar-Furth rats.

Objective: To determine whether BPA exposure of dams during gestation only or throughout lactation affects the incidence of mammary gland neoplasia in offspring.

Methods: We treated pregnant Sprague-Dawley rats with 0, 0.25, 2.5, 25, and 250 μg BPA/kg BW/day from gestational day (GD)9 to birth and from GD9 to postnatal day (PND)21. Mammary glands from dosed offspring were examined at four timepoints for preneoplastic and neoplastic lesions. To assess circulating BPA levels, we exposed pregnant rats to vehicle or 250 μg BPA/kg BW/day during gestation only or during gestation/lactation. Sera from dams, fetuses and nursing pups were analyzed for unconjugated and total BPA.

Results: Total and unconjugated BPA were detected in 100% of dams and fetuses and 33% of pups exposed to 250 μg BPA/kg BW/day. Unconjugated BPA levels in exposed dams and fetuses (gestational) and in exposed dams and pups (gestational/lactational) were within levels found in humans. Preneoplastic lesions developed in BPA-exposed rats across all doses as early as PND50. Unexpectedly, mammary gland adenocarcinomas developed in BPA-exposed females by PND90.

Conclusions: Developmental exposure to environmentally relevant levels of BPA during gestation and lactation induces mammary gland neoplasms in the absence of any additional carcinogenic treatment. Thus, BPA may act as a complete mammary gland carcinogen.

INTRODUCTION

Cumulative exposure to ovarian steroids during a woman's lifetime represents the most well-defined risk factor for the development of breast cancer. Epidemiological studies suggest that increased estrogen levels in the fetal environment are associated with an increased risk of breast cancer during adult life (Braun et al. 1995; Ekbom et al. 1992; Potischman and Troisi 1999). The synthetic estrogen diethylstilbestrol (DES), prescribed from the 1940s to 1970s to prevent miscarriage, is recognized as a seminal example of a human transplacental carcinogen for the multitude of adverse effects manifested in adult offspring. These effects include increased risk of vaginal clear cell carcinoma, reproductive tract malformations, poor pregnancy outcomes, compromised immune systems (Herbst et al. 1971; Hoover et al. 2011) and increased risk of developing breast cancer after 40 years of age (Hoover et al. 2011; Troisi et al. 2007). Another synthetic estrogen, bisphenol A (BPA), is currently one of the highest volume chemicals produced worldwide, with a global production capacity of 11.5 billion pounds in 2008 (Burridge 2008; vom Saal et al. 2007). BPA is used in the production of polycarbonate plastics, epoxy resins, dental sealants and composites, and thermal receipt paper. Incomplete polymerization of BPA leads to leaching of the chemical and subsequent human exposure, as evidenced by the detection of BPA in human urine, serum, maternal and fetal plasma, amniotic fluid, placenta, and adipose tissue (Calafat et al. 2005; Fernandez et al. 2007; Ikezuki et al. 2002; Schonfelder et al. 2002; Vandenberg et al. 2007a; Zalko et al. 2011). Although the oral exposure through ingestion of food and beverages was considered the main route in humans, recent publications indicate that humans are also exposed to BPA via inhalation, as well as via absorption through the skin and mucosal membranes of the mouth, and that these routes are not negligible (Vandenberg et al.

2013). The US Environmental Protection Agency (EPA) has calculated an oral reference dose for BPA of 50 μg BPA/kg body weight/day (μg BPA/kg BW/d) based on a lowest observed adverse effect level of 50 mg/kg BW/d (EPA 1993; Welshons et al. 2003). A review of more than two dozen biomonitoring studies that used analytical chemistry methods to measure BPA in healthy adults reported the detection of mean unconjugated BPA levels in the range of 1 ng/mL in blood (Vandenberg et al. 2010). The distinction between unconjugated and conjugated BPA is especially important in blood because the unconjugated form is considered the active form and has estrogenic activity (Thomas and Dong 2006; Watson et al. 2005). Recent pharmacokinetic analyses in non-human primates suggest that daily oral exposure to 400 μg BPA/kg BW/d is sufficient to produce serum concentrations of unconjugated BPA in the range measured in humans (Taylor et al. 2011), and that prenatal exposure to this dose altered the developing mammary glands of female rhesus monkeys (Tharp et al. 2012).

Fetal exposure to low doses of BPA altered the development of the rodent mammary gland, which manifested from the time of exposure and was exacerbated at puberty and beyond (Markey et al. 2001; Munoz de Toro et al. 2005; Vandenberg et al. 2007b; Vandenberg et al. 2008). Perinatal exposure to BPA increased estrogen and progesterone sensitivity in the mouse mammary gland (Ayyanan et al. 2011; Wadia et al. 2007). Altogether, these results suggest that perinatal exposure to BPA may increase the propensity to breast carcinogenesis. Supporting this assessment, rats exposed prenatally to 2.5 μg/kg BW/d BPA showed a significant increase in the number of mammary gland intraductal hyperplasias at PND50 and 90 compared to controls, and exposure to 250 or 1000 μg BPA/kg BW/d resulted in the development of ductal carcinomas *in situ* (DCIS) (Murray et al. 2007). Rats exposed prenatally to 25 μg BPA/kg BW/d displayed a higher number of ductal hyperplasias associated with desmoplasia in adulthood (Durando et al.

2007). Also, nitrosomethylurea administered at 50-days of age at doses that fail to induce tumors in control animals elicited the development of mammary carcinomas in females perinatally exposed to BPA (Durando et al. 2007). When rats or mice exposed perinatally to BPA were given the chemical carcinogen dimethylbenzanthracene (DMBA) during adulthood, this also resulted in increased tumor incidence and decreased tumor latency compared with animals exposed to DMBA alone (Jenkins et al. 2009; Weber Lozada and Keri 2011); this suggests that BPA can shift the window of susceptibility to DMBA (Betancourt et al. 2010). Additionally, lactational exposure to BPA between PND2 and 20 also increased tumor incidence in conjunction with a DMBA challenge (Jenkins et al. 2009).

As a follow-up to our previous work in Wistar-Furth (WF) rats (Murray et al. 2007), we examined the effect of duration of BPA exposure over a wide range of concentrations on the induction of preneoplastic lesions and DCIS in Sprague-Dawley (SD) rats, a strain used extensively for toxicology and carcinogenesis studies by the National Toxicology Program at the National Institute of Environmental Health Sciences. To relate this evidence to human biomonitoring data, we measured the internal levels of BPA in serum of these rats. Unexpectedly, at scheduled sacrifice times, we observed large mammary carcinomas (greater than 1cm^2 in diameter) occurring at internal doses relevant to human exposure, suggesting that BPA may act as a complete carcinogen.

MATERIALS AND METHODS

Animals

Sexually mature virgin female SD rats (8-10 weeks of age, Taconic, Germantown NY) were maintained in temperature and light controlled (14 h light, 10 h dark cycles) conditions in the

Tufts University School of Medicine Division of Laboratory Animal Medicine. Experimental procedures were approved by the Tufts University–Tufts Medical Center Animal Research Committee and all animals were treated humanely and with regard for alleviation of pain in accordance with the Guide for Care and Use of Laboratory Animals. Cages, water bottles and bedding tested negligible for estrogenicity by the E-SCREEN assay (Soto et al. 1992). Food (Harlan Teklad 2018) was supplied *ad libitum*. Estrogenicity of the feed was measured at 8-15 femtomoles of estrogen equivalents per gram, a negligible amount (Soto et al. 1992). Female rats were mated with SD males. The morning on which sperm was observed in vaginal smears was designated gestational day (GD) 1.

Fetal and Neonatal Exposure to BPA

In order to explore its role on neoplastic development, we administered BPA subcutaneously via Alzet osmotic pumps (Durect Corp., Cupertino, CA) with the dose calculated based on the weight of the dam at day 7 of pregnancy. Dams (N=9-12/dose/exposure period) were implanted with pumps on day 9 of pregnancy to administer vehicle (50% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO) or 0.25, 2.5, 25, or 250 µg BPA/kg BW/d. For convenience, these doses are subsequently referred to as BPA0.25, BPA2.5, BPA25, or BPA250, respectively.

We examined two different exposure periods. For animals exposed only through gestation, dams were implanted with pumps (cat#2002) designed to deliver continuously up to 14 days (see Supplemental Material, Figure S1A). These were implanted after 24 h equilibration as per manufacturer's specifications. For animals exposed through gestation and lactation, dams were implanted with pumps (cat#2004) designed to deliver continuously up to 28 days (see Supplemental Material, Figure S1B). These were implanted after 48 h of equilibration. In both

groups, animals delivered normally and litters were culled to 10 individuals on PND2. We distributed all female offspring (N=9-12/dose/age at sacrifice/exposure period) so that each litter was represented only once. We harvested mammary gland tissue at PND50, PND90, PND140 and PND200. The fourth left inguinal mammary gland was fixed and processed for paraffin embedding and the contralateral gland was whole mounted and stained with carmine as previously described (Murray et al. 2007).

Histological and Whole Mount Analysis

Three 5 μm sections separated by 50 μm were used to assess the presence of preneoplastic and neoplastic lesions in mammary glands of PND50 females. Five animals were sampled per dose from each exposure group for these analyses. We visualized histological sections with an Axioskop 2 Plus microscope (Carl Zeiss, München-Hallbergmoos, Germany) and captured images with an AxioCAm HR color digital camera (Carl Zeiss) and Axiovision software. We assessed the incidence of total ductal hyperplasia as previously described (Murray et al. 2007). Briefly, the leading edge and terminal end buds (TEBs) were localized and starting 400μm from the most proximal TEB, a 4 mm^2 box was drawn; all the ducts located within this area were counted. Usual intraductal hyperplasia (UDH) was characterized by an increase in monomorphic ductular epithelial cell layers (≥3 cells thick) typified by cuboidal or columnar pseudostratified epithelial cells that maintained a perpendicular orientation around the basement membrane. Preneoplastic lesions (atypical ductal hyperplasia; ADH) were characterized by an increase in monomorphic to mildly pleomorphic ductular epithelial cell layers, typified by flattened cuboidal epithelial cells that more intensely exhibited eosinophilic cytoplasmic staining and/or slightly enlarged hyperchromatic or vesicular nuclei. Neoplastic lesions (DCIS) were distinguished from

ADH by increased layers of disorganized, pleomorphic ductular epithelial cells bridging and occluding the lumen but maintaining an intact basement membrane (Davis and Fenton 2013).

Whole mounts of mammary glands harvested at PND50, PND90, PND140, and PND200 (N=9-12/treatment/age/exposure group) were assessed for proliferative lesions. Whole mounts were viewed with a stereomicroscope Stemi 2000 (Carl Zeiss). Detected lesions (<10 mm in diameter) and tumors (≥10 mm in diameter) were excised, sectioned, and H&E stained for diagnosis.

Quantification of circulating levels of BPA

A pilot experiment determined the levels of detectability for the assay to measure BPA in serum of pregnant dams. Based on dam weight at GD7, pregnant animals (N=6/dose) were implanted with pumps on GD9 to deliver vehicle, BPA25, or BPA250. We collected blood by cardiac puncture 72 hours later. All analyses of total and unconjugated serum BPA were conducted at the Centers for Disease Control and Prevention (Ye et al. 2008). Briefly, serum was either treated with β-glucuronidase/sulfatase to estimate the concentration of total BPA (conjugated plus unconjugated), or processed without enzymatic treatment to estimate the concentration of unconjugated BPA. Then, serum concentrations were quantified using on-line solid phase extraction coupled to high performance liquid chromatography-isotope dilution tandem mass spectrometry. The limit of detection (LOD), the lowest amount of an analyte that can be detected with a defined probability, was 0.3 ng/mL. The limit of quantification (LOQ), calculated as three times the LOD, defines the point at which data attain statistical significance.

In pregnant dams, total serum BPA was undetectable in all vehicle treated dams (N=6) and detectable, although below the limit of quantification of 0.9 ng/mL, in 50% of dams treated with BPA25 (mean±SD=0.37±0.27 ng/mL; N=6). Unconjugated BPA was detected in 100% of dams

(N=6) treated with BPA250 (total BPA=3.45±2.63 ng/mL; unconjugated BPA=0.83±0.31 ng/mL). Based on these results, we conducted a second experiment using either vehicle or BPA250 and divided the pregnant animals into two groups. One group was designed to measure fetal exposure near the end of gestation to ensure the collection of a sufficient volume of fetal serum; dams were weighed at GD17 to calculate the BPA dose administered, and animals were implanted at GD18 with cat#2002 pumps. This group corresponds to the gestational-only exposure group described above. Animals were killed on GD21 and serum was collected from each dam and fetal sera from each litter were pooled (see Supplemental Material, Figure S1C). The second group was designed to measure BPA levels in dams and pups at PND10; dams were weighed at GD7 and implanted with #2004 pumps on GD9. This corresponds to the gestational/lactational exposure group described above. Animals delivered normally and litters were culled to 10 on PND2. Dams and pups were killed on PND10 and the serum collected (see Supplemental Material, Figure S1D). Serum of the pups was pooled by litter.

Statistical Analyses

All calculated parameters and statistical significance were determined using SPSS statistical software (SPSS, Chicago, IL). For the statistical calculations involving total and unconjugated BPA concentrations, we used the instrument generated values, even if they were below the LOD, to run student *t*-tests. All results are presented as mean±SD. Overall differences in preneoplastic lesions were analyzed by ANOVA, and differences in tumor incidence were analyzed via chi-square. For all statistical tests, results were considered significant at $p<0.05$.

RESULTS

BPA in serum of dams and their offspring following continuous exposure to BPA

BPA was mainly present in its conjugated form in the serum of dams, fetuses and pups. Total and unconjugated BPA serum concentrations were detectable in 100% of pregnant dams and fetuses exposed gestationally to BPA250. Mean serum concentration of total BPA was significantly higher in exposed fetuses compared to their vehicle controls, whereas the difference between mean unconjugated BPA levels between exposed fetuses and their controls approached significance ($p=0.051$; Table 1). Unconjugated BPA was significantly higher in exposed dams compared to controls. The average total BPA in the serum of exposed fetuses was four-times greater than in the serum of the dams ($p=0.004$). Total and unconjugated serum BPA were detectable in 100% of lactating dams but total BPA was detectable in only 33% of PND10 pups exposed gestationally/lactationally to BPA (Table 2). Serum concentrations of total and unconjugated BPA were significantly higher in exposed lactating dams compared to controls (Table 2). Total serum BPA measured in exposed pups was significantly higher than in controls; however all values were below the LOQ. The mean total BPA concentration in the exposed pups (0.38 ng/mL) was significantly lower than in their respective dams (16.50 ng/mL; $p=0.03$). Because BPA is ubiquitously present in the environment, extensive measures were taken throughout the process from blood sampling to final assay to reduce contamination. Detection of predominantly conjugated versus unconjugated BPA in serum of dams, fetuses and pups suggests that external contamination, if it occurred, was not systematic or extensive.

Preneoplastic and neoplastic lesions developed in perinatally exposed mammary glands

We analyzed histological sections at PND50 for incidence of preneoplastic and neoplastic lesions (Figure 1). The incidence of UDH was not significantly different between the BPA and vehicle-treated animals for either exposure period (data not shown). However, histological assessment of glands for ADH or DCIS showed that the incidence of ADH among glands from females exposed to all doses of BPA during both exposure times ranged from 0-60% compared to 0% incidence in the vehicle-treated animals (Table 3). We diagnosed DCIS in a gland from a female exposed gestationally/lactationally to BPA25 (Figure 1D).

We observed mammary gland lesions in whole mounts by PND90 (Figure 2). Four animals exhibited proliferative lesions in their mammary glands at PND90 and PND140 following either gestational-only or gestational/lactational exposure. One lesion in a vehicle-exposed gland at PND140 was diagnosed as a benign microfibroadenoma. The three remaining mammary gland lesions were observed in three different females each exposed to BPA0.25, 25, or 250; all were diagnosed as lobular alveolar hyperplasia (Table 4).

Malignant tumors developed following perinatal exposure to BPA

We detected tumors at PND90, PND140 and PND200 in animals exposed to BPA across all doses and exposure times. Six mammary gland tumors in total were observed in females exposed perinatally to BPA at doses ranging from BPA0.25 to BPA250 (N=230; Table 4). Five tumors were diagnosed histopathologically as adenocarcinomas and one was diagnosed as a benign fibroadenoma (Figure 3). No malignant tumors were detected in any control animals (N=65).

DISCUSSION

Developmental exposure to BPA was shown to increase the propensity to mammary gland carcinogenesis in rodents (Betancourt et al. 2010; Durando et al. 2007; Jenkins et al. 2009; Murray et al. 2007; Weber Lozada and Keri 2011). In the present study, perinatal exposure to human relevant internal doses of BPA, in the absence of additional exposure to chemical carcinogens, led to the induction of malignant mammary gland tumors and other lesions in adult female rats. In order to correlate dose and effect, we assessed the internal BPA dose in exposed dams and their offspring. Gestational exposure to BPA250 resulted in the detection of unconjugated BPA in both dams and fetuses at levels significantly higher than in controls, within the range of the levels detected in human serum (Table 1). This validates the use of osmotic pumps as an effective route of BPA administration to the fetus. Remarkably, the average total BPA measured in fetuses at GD21 following gestational exposure to BPA was four-times greater than in dams. Although UDP-glucuronosyltransferase-2B1, the major liver enzyme responsible for conjugation (inactivation) of BPA via glucuronidation, shows little to no activity in the rat fetal liver (Yokota et al. 1999), the concentrations of glucuronidated BPA in the placenta and fetus have been shown to be higher than in maternal blood following administration of BPA to dams (Takahashi and Oishi 2000; Zalko et al. 2003).

Total and unconjugated BPA concentrations measured in lactating dams at PND10 following gestational/lactational BPA exposure were higher than in their nursing pups (Table 2). In fact, unconjugated BPA was undetectable in all the pups examined, while total BPA was detected in 33% of the BPA-exposed pups. These results suggest that when a dam is continuously exposed to a constant dose of BPA during gestation and lactation, the neonate is exposed to lower levels of BPA than the fetus probably due to the lack of transfer in milk. Comparable results were

reported with serum concentrations of unconjugated BPA undetectable and total BPA approximately 300-times lower in suckling rat pups relative to their dams (Doerge et al. 2010); these authors attributed the low plasma levels observed in pups to low BPA intake from the mother's milk. Applying this relationship to our internal dose study, one may conclude that the pups from the BPA250 dams were lactationally exposed to 0.8 µg total BPA/kg BW/d, below the current US EPA reference dose (EPA 1993). The increase in BPA levels in the dams at PND10 and the significant decrease in BPA in the pups compared to fetuses could also be attributed to maternal grooming practices, as suggested for other chemicals (White et al. 2007).

Oral administration of tritiated BPA has been shown to result in a linear relationship between the administered dose and unconjugated serum BPA concentrations in both rodents and primates (Taylor et al. 2011). In the present study, the mean unconjugated serum BPA concentration in dams exposed either gestationally or gestationally/lactationally to BPA250 was 1.25 ng/mL even though the mean total BPA concentrations were almost three times higher in dams exposed gestationally/lactationally than only gestationally. Assuming linearity of circulating levels with dose, the concentrations of unconjugated BPA following exposure to BPA0.25, BPA 2.5, and BPA25 could be estimated around 0.00125, 0.0125, and 0.125 ng/mL, respectively, levels below the LOD of our method (0.3 ng/mL). However, at these undetectable levels, we have clearly seen effects in the mammary gland, both in the present data set and in our previous study (Murray et al. 2007).

Earlier studies provided evidence that perinatal exposure to low doses of BPA resulted in altered morphogenesis of the rodent mammary gland that first manifests during the exposure period. As the gland undergoes further changes under the influence of ovarian and pituitary hormones, morphological alterations become more pronounced after the onset of puberty and throughout

adulthood (Munoz de Toro et al. 2005; Vandenberg et al. 2007b). In the current study, non-neoplastic lesions diagnosed as lobular alveolar hyperplasia were observed in whole mounts of mammary glands at PND90 and PND140. These lesions have been associated with the administration of xenobiotics that act as estrogen receptor (ER) agonists (Biegel et al. 1998) or dopaminergic receptor antagonists that cause prolactinemia in female rats (Lotz and Krause 1978; Lucas et al. 2007). A previous study showed that a single injection of the carcinogen N-nitrosomethylurea to post-pubertal virgin female rats resulted in secretion of α-lactalbumin from lobular alveolar structures in the PND200 mammary gland (Murray et al. 2009). Therefore, the increased secretory material in the ducts and/or alveoli in the lesions observed in the present study may be due to xenobiotic-induced prolactinemia.

In this study, we examined whether exposure to environmentally relevant doses of BPA influenced the development of preneoplastic and neoplastic lesions in SD rats. Unlike our previous study in WF rats (Murray et al. 2007), there was no difference in the incidence of UDH in SD females exposed to BPA compared to controls. This could be attributed to strain differences in the overall histoarchitecture of the mammary gland in the peripubertal phase [Fenton S, personal communication]. Alternatively, the time-course of development and regression of these lesions may be different in these strains. In the present study, both ADH and DCIS were identified only in glands of females exposed to BPA, at doses as low as BPA0.25.

To our knowledge, the induction of malignant tumor formation following developmental exposure to environmentally relevant levels of BPA has not been previously reported. As an unexpected outcome of the present study, adenocarcinomas were identified in females as young as PND90 and at doses as low as BPA0.25. It is important to note that no control animals developed malignant tumors throughout the duration of the study. Historical data on the natural

occurrence of neoplastic lesions in control female SD rats from large carcinogenicity trials established that there is evidence that spontaneous malignant tumors do not occur before PND210, and in fact most occurred after PND350 (Ikezaki et al. 2011; National Toxicology Program 2008; National Toxicology Program 2010; Son and Gopinath 2004). Although the incidence of tumor development was not statistically significant, the highly adverse nature of carcinomas validates the biological importance of reporting this outcome, as this information warrants further studies.

How BPA contributes to the initiation and progression of neoplasia is still unknown; however, perinatal exposure to low doses of BPA has been shown to affect the hypothalamic-pituitary-ovarian axis via a) altered development of the hypothalamic nuclei essential for cyclic gonadotropin release (Rubin et al. 2006), b) disrupted estrous cyclicity of exposed individuals (Rubin et al. 2001), and c) increased sensitivity of the adult mammary gland to ovarian hormones (Ayyanan et al. 2011; Munoz de Toro et al. 2005). It is likely that BPA, like DES, may induce carcinogenesis by acting as an estrogen. During fetal life, ERs α and β are only present in stromal cells; epithelial ER expression begins at the end of gestation (Vandenberg et al. 2007b). Low-dose BPA exposure (250 ng/kg BW/d) during mouse fetal development altered the composition and organization of the extracellular matrix (Wadia et al. 2013) and accelerated maturation of the presumptive fat pad, an event necessary for ductal invasion and branching. This suggests that BPA acts directly on the stroma, which may in turn alter the development of the epithelium as evidenced by increased ductal area and delayed lumen formation during the period of exposure (Vandenberg et al. 2007b). These data are compatible with the tissue organization field theory of carcinogenesis that posits that carcinogens alter the reciprocal interactions between stroma and

epithelium (Soto and Sonnenschein 2011), as shown by tissue recombination studies (Maffini et al. 2004).

According to the US EPA, a carcinogen is a chemical or physical agent capable of causing cancer, a definition that does not specify the mechanism(s) by which the cancer is induced; it only identifies the consequence of an insult (EPA 2005). Thus, by this definition, and by the data provided in these studies, BPA may act as a complete mammary gland carcinogen.

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Table 1. Internal dose of BPA measured in SD dams and their fetuses at GD21 following continuous exposure during gestation only (GD18-GD21).

BPA dose (µg/kg BW/d)	Animals	Incidence of Detectable BPA (T)	Mean BPA (T) (ng/mL)	RangeBPA (T) (ng/mL)	Incidence of Detectable BPA (U)	Mean BPA (U) (ng/mL)	RangeBPA (U) (ng/mL)	Ratio (T:U) BPA
0	Dams	3/6[a]	<LOD	0.0 - 0.4[b]	0/6	<LOD	0.0 - 0.2 [b]	N/A
0	Fetuses	0/5	<LOD	0.1 – 0.2 [b]	0/5	<LOD	0.0 - 0.05 [b]	N/A
250	Dams	4/4	6.13±3.88	2.5 - 11.4	4/4	1.25±0.10*	1.2 – 1.4	5:1
250	Fetuses	4/4	27.90±8.96*	16.7 - 36.7	4/4	0.63±0.39	0.4 – 1.7 [c]	44:1

GD= gestational day; T=total; U=Unconjugated

[a]: the number of animals with BPA measurements at or above the limit of detection (LOD, the lowest amount of BPA that can be detected with a defined probability, 0.3 ng/mL).

[b]: all concentrations were at or below the limit of quantification (0.9 ng/mL; the lowest amount of BPA that can be quantitatively determined with suitable precision and accuracy).

[c]:at least one or more concentrations were at or below the limit of quantification (0.9 ng/mL)

Values expressed as mean±SD.

Student *t*-tests were used to compare treated groups to controls.

*= significant treatment effect compared to control; $p<0.05$. n=4-6 animals/treatment group.

Table 2. Internal dose of BPA measured in SD lactating dams and their nursing pups at PND10 following continuous exposure during gestation/lactation (GD9-PND10).

BPA dose (µg/kg BW/d)	Animals	Incidence of Detectable BPA (T)	Mean BPA (T) (ng/mL)	Range BPA (T) (ng/mL)	Incidence of Detectable BPA (U)	Mean BPA (U) (ng/mL)	Range BPA (U) (ng/mL)	Ratio (T:U) BPA
0	Dams	2/6 [a]	0.35±0.43	0 – 0.9 [b]	0/6	<LOD	0.0 – 0.3 [b]	N/A
0	Pups	0/6	<LOD	0 – 0.1 [b]	0/6	<LOD	N/A	N/A
250	Dams	6/6	16.50±13.00*	6 – 38.8	6/6	1.25±0.44*	0.9 – 2.1[c]	13:1
250	Pups	2/6 [a]	0.38±0.26*	0.2 – 0.8 [b]	0/6	<LOD	0.0 – 0.1 [b]	N/A

PND=post-natal day; GD= gestational day; T=total; U=Unconjugated

[a]: the number of animals with BPA measurements at or above the limit of detection (LOD, the lowest amount of BPA that can be detected with a defined probability, 0.3 ng/mL).

[b]: all concentrations were at or below the limit of quantification (0.9 ng/mL; the lowest amount of BPA that can be quantitatively determined with suitable precision and accuracy).

[c]:at least one or more concentrations were at or below the limit of quantification (0.9 ng/mL)

Values expressed as mean±SD.

Student *t*-tests were used to compare treated groups to controls.

*= significant treatment effect compared to control; $p<0.05$. $n=6$ animals/treatment group.

Table 3. Preneoplastic and neoplastic lesions observed at PND50 in histological sections of mammary glands of BPA-exposed females.

BPA dose (µg/kg BW/d)	Incidence Gestational Exposure N (%)	Diagnosis	Incidence Gestational/Lactational Exposure N (%)	Diagnosis
0	0/5 (0%)	N/A	0/5 (0%)	N/A
0.25	3/5 (60%)	ADH	0/5 (0%)	N/A
2.5	1/5 (20%)	ADH	1/5 (20%)	ADH
25	0/5 (0%)	N/A	1/5 (20%)	DCIS; ADH
250	2/5 (40%)	ADH	1/6 (17%)	ADH

ADH = atypical ductal hyperplasia; DCIS = ductal carcinoma *in situ.*

N/A = no lesion detected for diagnosis.

Differences in observed lesions following treatment were analyzed by ANOVA, and found not to be significant.

Table 4. Proliferative mammary gland lesions and tumors observed at PND90, 140, and 200 following either gestational only or gestational/lactational exposure to BPA.

	Incidence (Gestational Exposure)			Incidence (Gestational/Lactational Exposure)		
BPA dose (µg/kg BW/d)	**Total lesions**	**Cancer**	**Diagnosis (Age)**	**Total lesions**	**Cancer**	**Diagnosis (Age)**
0	1/35	0/35	Microfibroadenoma[a] (PND140)	0/30	0/30	N/A
0.25	1/31	1/31	Adenocarcinoma[bc] (PND200)	1/27	0/27	Lobular alveolar hyperplasia[a] (PND140)
2.5	1/28	1/28	Adenocarcinoma[bc] (PND90)	2/30	1/30	Adenocarcinoma[bc] (PND140); Fibroadenoma[ac] (PND200)
25	0/23	0/23	N/A	2/28	1/28	Lobular alveolar hyperplasia[a] (PND90); Adenocarcinoma[bc] (PND140)
250	2/30	1/30	Lobular alveolar hyperplasia[a] (PND90); Adenocarcinoma[bc] (PND140)	0/33	0/33	N/A

[a] Benign; [b] Malignant; [c] Tumor

Ratio = number of animals with lesion or tumor relative to the combined number of animals at PND90, 140, and 200 per dose per exposure period.

N/A = no lesion or tumor detected for diagnosis.

Differences in tumor incidence following treatment compared to vehicle were analyzed by chi-square, and found not to be significant.

Figure Legends

Figure 1. Preneoplastic and neoplastic lesions observed in PND50 mammary glands. (A) UDH; (B, C) ADH; and (D) DCIS. Scale bar = 50μm

Figure 2. Presumptive lesions detected in mammary gland whole mounts from PND90 (A, B= 1.7mm and 6.3mm, respectively) and PND140 (C= 5.8mm) females. Scale bar: 1 mm. Lesions were excised, sectioned, and H&E stained for diagnosis. One microfibroadenoma (benign) was diagnosed in a vehicle-treated female at PND140 (not shown). Lobular alveolar hyperplasia was characterized by infiltration of mammary fat pad with glandular acini (a) and/or by focal, irregular proliferation of alveolar epithelium (b,c) with secretory activity (arrowhead). Scale bar: 50μm.

Figure 3. H+E staining of tumors removed from BPA-treated rats at time of sacrifice. Tumors were detected at PND90, 140, and 200 in gestationally and gestationally/lactationally exposed females. No tumors were observed in vehicle-treated animals. Insets: Magnification 10x base image to show detail of tumor morphology. Scale bar: 500μm

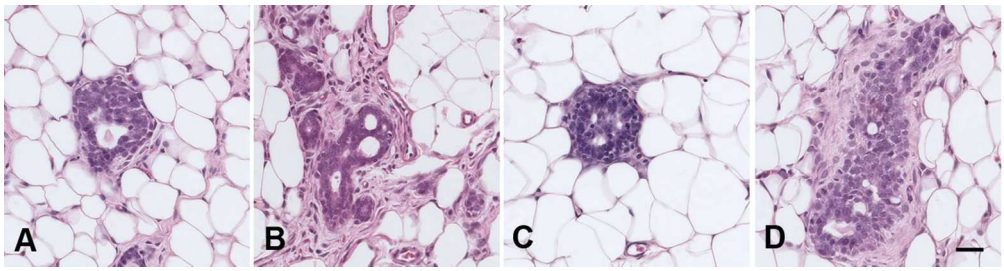

Figure 1
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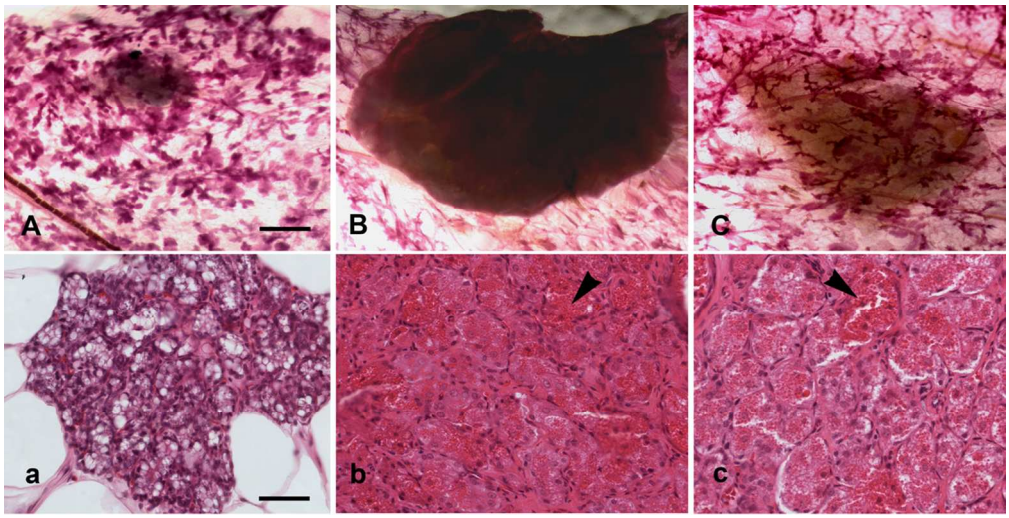

Figure 2
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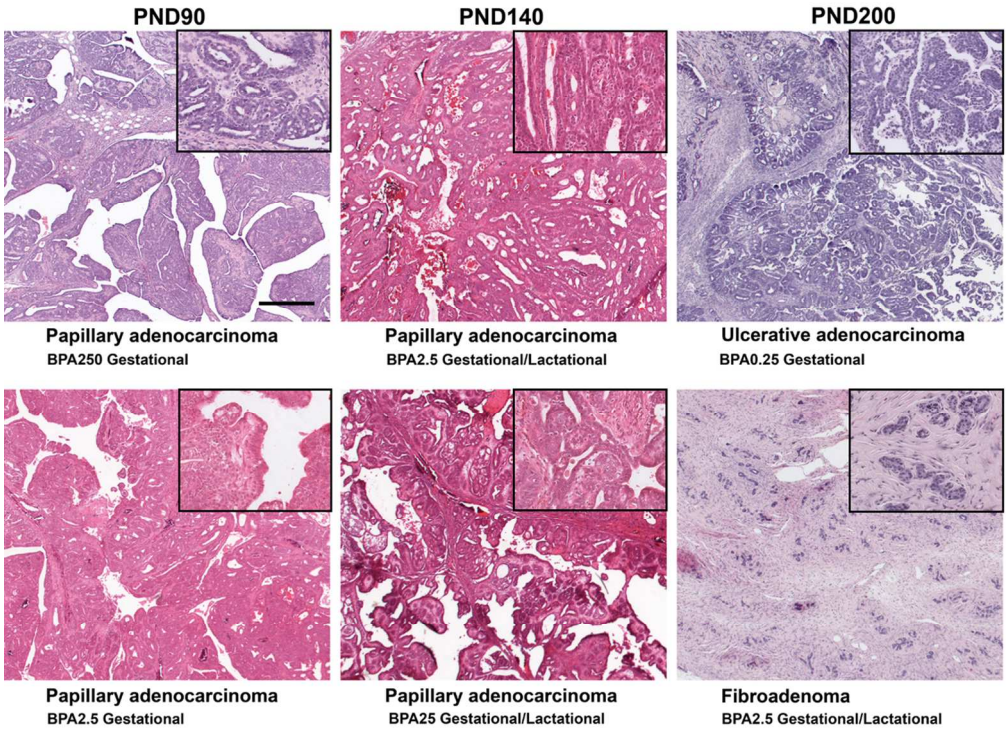

Figure 3
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